Associations Between *TIM1* Polymorphisms and Dilated Cardiomyopathy in a Han Chinese Population

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SUMMARY

Immune dysfunction is implicated in dilated cardiomyopathy (DCM). Previous studies found that *TIM1* polymorphisms were associated with immune dysfunction. However, the associations between *TIM1* polymorphisms and DCM have not been investigated. Therefore, we conducted the present study to evaluate whether *TIM1* polymorphisms were associated with DCM in the Han Chinese population. A total of 396 DCM patients and 403 healthy controls were enrolled in this case-control study. Two promoter region single nucleotide polymorphisms (SNPs) of *TIM1* gene, -416G>C and -1454G>A, were genotyped by PCR-RFLP. The associations between two SNPs genotyped and the overall survival (OS) of DCM patients were evaluated with Kaplan-Meier analysis and Cox regression analysis. Plasma TIM-1 levels were further measured by ELISA. We found that the C allelic frequency of -416G>C and A allelic frequency of -1454G>A were higher in DCM patients than that in controls ($P < 0.001$). The genotypic frequencies of both SNPs were associated with DCM susceptibility in the codominant, dominant, and overdominant models ($P < 0.01$). They were also associated with the OS of DCM patients in the dominant, recessive, and overdominant models ($P < 0.001$). The CC genotype of -416G>C and AA genotype of -1454G>A were associated with the worst prognosis ($P < 0.001$). In addition, the plasma TIM-1 levels in DCM patients were higher than that in controls (259.0 pg/mL versus 149.8 pg/mL, $P = 0.035$). The CC genotype of 416G>C and AA genotype of -1454G>A were associated with the highest TIM-1 production ($P < 0.01$). Overall, our findings suggest that *TIM1* polymorphisms are associated with DCM susceptibility and prognosis in this Han Chinese population. (Int Heart J 2016; 57: 742-746)

Key words: Single nucleotide polymorphisms, Survival analysis, Enzyme-linked immunosorbent assay

Dilated cardiomyopathy (DCM), characterized by progressive ventricular chamber enlargement and contractional dysfunction in the absence of associated conditions such as hypertension, ischemic heart disease, valvular heart disease, congenital heart disease, and pulmonary heart disease, is the most common type of cardiomyopathy, accounting for approximately 55-60% of cardiomyopathies with an estimated prevalence of 1 in 2,500 individuals.¹⁾ It is the leading non-ischemic cause of congestive heart failure and sudden cardiac death, and is the most frequent indication for cardiac transplantation in adults and children.²⁾

In the last two decades, extensive studies have been conducted to investigate the etiology of DCM. However, the exact cause of the disease is still largely unknown. Recently, immune dysfunction was demonstrated to be involved in the pathogenesis of DCM. Firstly, it was found that T suppressor (Ts) cells are decreased while T helper (Th) cells are increased in DCM patients.³⁾ Secondly, several cardiac-specific antibodies were detected in serum of DCM patients.⁴⁻⁷⁾ And thirdly, immunosuppressive therapy was shown to be associated with sympto-

matic and functional improvements of DCM patients in some pilot studies.^{8,9)} These findings jointly indicate that immune dysfunction is an important pathogenic factor of DCM.

T cell immunoglobulin domain and mucin domain-1 (TIM-1), the founding member of the TIM family, which is encoded by the *TIM1* gene located on chromosome 5q33.2, is a type 1 transmembrane glycoprotein comprised of an immunoglobulin variable domain, a heavily glycosylated mucin domain, a single transmembrane domain, and a cytoplasmic tail with one tyrosine phosphorylation motif.¹⁰⁾ Studies over the past several years have shown that TIM-1 plays a crucial role in regulating immune responses and maintaining immune homeostasis. In addition, accumulating evidence has demonstrated that immune dysfunction caused by certain *TIM1* polymorphisms is associated with autoimmune diseases, allergic reactions, chronic viral infections, and immunologic rejections.¹¹⁾

Therefore, we hypothesized that certain *TIM1* polymorphisms, which cause disturbance of the immune system, may contribute to the occurrence and development of DCM in some

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patients. In the present study, polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis and survival analysis were carried out to evaluate the associations of two single nucleotide polymorphisms (SNPs) located in the promoter region of the *TIM1* gene (-416G>C and -1454G>A) with DCM susceptibility and prognosis. In addition, enzyme-linked immunosorbent assay (ELISA) was performed to reveal the potential relationship between *TIM1* polymorphisms and TIM-1 protein production.

METHODS

Study subjects: In this case-control study, a cohort of 396 DCM patients (mean age, 49.0 ± 14.1 ; male/female, 246/150) was consecutively recruited from the West China Hospital of Sichuan University between June 2002 and September 2014. The diagnosis of DCM was made in accordance with the criteria established by the World Health Organization/International Society and Federation of Cardiology Task Force on the Classification of Cardiomyopathies in 1995 (before 2006) and the scientific Statement on the Definitions and Classification of Cardiomyopathies proposed by the American Heart Association in 2006 (after 2006).^{1,12} A total of 403 healthy individuals (mean age, 49.5 ± 13.1 ; male/female, 248/155) from a routine physical examination were enrolled as controls. All subjects were of Han Chinese ethnicity living in Sichuan province, which is located in southwest China. Patients with a history of hypertension, ischemic heart disease, valvular heart disease, congenital heart disease, pulmonary heart disease, tachyarrhythmia, pericardial disease, heavy alcohol consumption, acute viral myocarditis, systemic diseases of putative autoimmune origin, skeletal myopathies or nutrition disorders were intentionally excluded. The baseline characteristics of the study population are summarized in Supplemental Table I. The current study was performed with approval from the West China Hospital Ethics Committee and in accordance with the Declaration of Helsinki. Peripheral blood samples were obtained from all subjects after obtaining written informed consent.

Isolation of genomic DNA and genotyping of two SNPs: Genomic DNA was extracted from peripheral blood samples using a DNA isolation kit (Biotek, Beijing, China) and the procedure was performed according to the manufacturer's protocol. DNA samples were subsequently stored at -20°C for future use. Genotyping of the two *TIM1* SNPs, -416G>C and -1454G>A, was carried out using PCR-RFLP analysis. The primer pairs, F: 5'-TAACCGCCAACTGACTCCT-3' and R: 5'-TCACCCTTTTAGCCAGGATG-3', F: 5'-TCTTGAACCTCCGACCTCAG-3' and R: 5'-TTCATGGGGAAGTGGATGAT-3', were used to amplify the genomic region surrounding -416G>C and -1454G>A, respectively. The PCR reaction was performed in a total volume of 25 μL solution, containing 2.5 μL 10 $\times$ PCR buffer, 1.5 mmol/L MgCl_2 , 0.15 mmol/L dNTPs, 0.5 $\mu\text{mol/L}$ each primer, 100 ng genomic DNA, and 2U of Tag DNA polymerase (NEB, Ipswich, MA, USA). The PCR cycling conditions consisted of an initial denaturation step at 94°C for 4 minutes, followed by 36 cycles of denaturation at 94°C for 30 seconds, annealing at 58°C (-416G>C) and 60°C (-1454G>A) for 30 seconds, extension at 72°C for 30 seconds, and a final elongation step at 72°C for 10 minutes. To avoid contamination, negative controls without genomic DNA were

included in each PCR reaction. PCR products were run on 6% polyacrylamide gels and visualized by means of silver staining to check whether amplification reactions were successful. After ensuring the success of PCR reactions, 3.5 μL of PCR products generated were digested with corresponding locus-specific restriction enzymes in a total volume of 10 μL solution for 2-3 hours (2U Taq^I at 65°C for -416G>C, 2U MspI at 37°C for -1454G>A) (NEB, Ipswich, MA, USA). The digested products were then separated on 6% polyacrylamide gels and stained with 1.5 g/L argent nitrate for genotypic determination. For genotyping of -416G>C polymorphism, a single uncuttable fragment of 167 bp indicated the GG genotype, the heterozygous GC genotype was indicated by 3 fragments of 167 bp, 141 bp and 26 bp, while the CC genotype was indicated by two fragments of 141 bp and 26 bp. For genotyping of the -1454G>A polymorphism, a single uncuttable fragment of 148 bp indicated the AA genotype, the heterozygous GA genotype was indicated by 3 fragments of 148 bp, 78 bp, and 70 bp, while the GG genotype was indicated by two fragments of 78 bp and 70 bp. About 10% of the samples were randomly selected for direct sequencing to test the accuracy of genotypic determination and the results were 100% concordant.

Clinical follow-up: A total of 265 patients who provided contact information in their medical records were arranged for regular follow-up at time intervals of 6-12 months. The clinical follow-up was carried out in a blind manner in regard to the patient's genotypes. The endpoint of follow-up was cardiac death or loss of contact.

Enzyme-linked immunosorbent assay: Plasma was obtained from peripheral blood samples after centrifuging at 2,000 rpm for 10 minutes, and was stored at -80°C until further analysis. To measure plasma levels of TIM-1 in DCM patients and healthy controls, a pre-coated human TIM-1 ELISA kit (Raybiotech, Norcross, GA, USA) was used according to the manufacturer's protocol. The minimum detectable TIM-1 level was 2 pg/mL.

Statistical analysis: All data analyses were performed using SPSS 19.0 statistical software for Windows (SPSS Inc, Chicago, IL, USA). Genotypic and allelic frequencies of the two SNPs genotyped were obtained by directed counting. Hardy-Weinberg equilibrium (HWE) in the control group was evaluated with the chi-square test. The associations between two SNPs genotyped and DCM susceptibility were assessed in the codominant, dominant, recessive, and overdominant models using SNPstats. Odds ratios (ORs) and 95% confidence intervals (95% CIs) were utilized to evaluate the effects of any differences in the distribution of genotypes and alleles. Kaplan-Meier analysis and Cox regression analysis were further performed to test the correlations between two SNPs genotyped and overall survival (OS) of DCM patients. ELISA data were analyzed with the Pearson correlation test and Mann-Whitney *U* test. The Kruskal-Wallis test was subsequently carried out to explore the potential associations of two SNPs genotyped with plasma TIM-1 and brain natriuretic peptide (BNP) levels. A raw probability value (*P* value) of 0.05 or less was considered as statistically significant, which was further subjected to Bonferroni correction to account for multiple comparisons. The significance threshold was set at 0.025 for a single SNP test since two SNPs were analyzed.

Table. Distributions of *TIM1* SNPs Among Cases and Controls and Their Associations With DCM Susceptibility

		-416G>C (rs9313422)				-1454G>A (rs41297579)					
		Cases, <i>n</i> (%)	Controls, <i>n</i> (%)	OR (95%CI)	<i>P</i>			Cases, <i>n</i> (%)	Controls, <i>n</i> (%)	OR (95%CI)	<i>P</i>
Model	Genotype					Genotype					
Codominant	GG	159 (40.1%)	213 (52.9%)	1.00	0.0015	GG	271 (68.4%)	318 (78.9%)	1.00	0.0021	
	GC	194 (49%)	156 (38.7%)	1.67 (1.23-2.22)		GA	106 (26.8%)	76 (18.9%)	1.64 (1.16-2.27)		
	CC	43 (10.9%)	34 (8.4%)	1.69 (1.03-2.78)		AA	19 (4.8%)	9 (2.2%)	2.50 (1.10-5.56)		
Dominant	GG	159 (40.1%)	213 (52.9%)	1.00	<0.001	GG	271 (68.4%)	318 (78.9%)	1.00	<0.001	
	GC/CC	237 (59.9%)	190 (47.1%)	1.67 (1.27-2.22)		GA/AA	125 (31.6%)	85 (21.1%)	1.72 (1.25-2.38)		
Recessive	GG/GC	353 (89.1%)	369 (91.6%)	1.00	0.25	GG/GA	377 (95.2%)	394 (97.8%)	1.00	0.046	
	CC	43 (10.9%)	34 (8.4%)	1.32 (0.83-2.13)		AA	19 (4.8%)	9 (2.2%)	2.22 (0.99-5.00)		
Overdominant	GG/CC	202 (51%)	247 (61.3%)	1.00	0.0034	GG/AA	290 (73.2%)	327 (81.1%)	1.00	0.0076	
	GC	194 (49%)	156 (38.7%)	1.52 (1.15-2.00)		GA	106 (26.8%)	76 (18.9%)	1.56 (1.12-2.17)		
	Allele					Allele					
	G	512 (64.6%)	582 (72.2%)	1.00		<0.001	G	648 (81.8%)	712 (88.3%)		1.00
	C	280 (35.4%)	224 (27.8%)	1.43 (1.15-1.75)			A	144 (18.2%)	94 (11.7%)		1.67 (1.27-2.22)

RESULTS

In the present study, two SNPs located in the promoter region of the *TIM1* gene, -416G>C and -1454G>A, were genotyped in 396 DCM patients and 403 healthy controls. The distributions of the two SNPs were both in accordance with HWE ($P > 0.05$) in the control group, indicating that the genotypic and allelic frequencies of the two SNPs met the expected equilibrium and thus were representative. The associations between the two SNPs genotyped and DCM susceptibility were determined by comparing genotypic and allelic frequencies in DCM patients and healthy controls. As shown in the Table, statistically significant differences were found for both SNPs. The genotypic frequency of -416G>C was associated with DCM susceptibility in the codominant model [OR = 1.67 (1.69), 95%CI 1.23-2.22 (1.03-2.78), $P = 0.0015$], the dominant model (OR = 1.67, 95%CI 1.27-2.22, $P < 0.001$), and the overdominant model (OR = 1.52, 95% CI 1.15-2.00, $P = 0.0034$). The C allelic frequency in DCM patients and healthy controls was 35.4% and 27.8%, respectively, which was significantly higher in DCM patients than that in healthy controls (OR = 1.43, 95%CI 1.15-1.75, $P < 0.001$). Similarly, the genotypic frequency of -1454G>A was also associated with DCM susceptibility in the codominant model [OR = 1.64 (2.50), 95%CI 1.16-2.27 (1.10-5.56), $P = 0.0021$], the dominant model (OR = 1.72, 95%CI 1.25-2.38, $P < 0.001$), and the overdominant model (OR = 1.56, 95%CI 1.12-2.17, $P = 0.0076$). The A allelic frequency in DCM patients and healthy controls was 18.2% and 11.7%, respectively, which was also significantly higher in DCM patients than that in healthy controls (OR = 1.67, 95%CI 1.27-2.22, $P < 0.001$).

The correlations between the two SNPs genotyped and prognosis of DCM patients were subsequently assessed in survival analysis. The 265 contactable DCM patients (mean age, 48.7 ± 14.2 ; male/female, 175/90) were screened for a mean period of 69.0 ± 20.4 months. During follow-up, all patients received continuous medication treatment and none underwent heart transplantation. At the end of the present study, 183 patients (69.1%) had suffered cardiac death. Kaplan-Meier analy-

sis revealed a significant association between both SNPs and the prognosis of DCM patients. Hazard ratios (HRs) and 95%CI were further evaluated in the Cox regression analysis with adjustment for age, sex, NYHA (New York Heart Association functional class), LVEDD (left ventricular end-diastolic diameter), LVEF (left ventricular ejection fraction), and BNP level. As shown in Supplemental Table II, the univariate survival analysis demonstrated that genotypic frequency of -416G>C was significantly associated with OS of DCM patients in the dominant model (HR = 3.26, 95%CI 2.37-4.49, $P < 0.001$), the recessive model (HR = 3.98, 95%CI 2.49-6.37, $P < 0.001$), and the overdominant model (HR = 2.16, 95%CI 1.59-2.94, $P < 0.001$). Patients with GG genotype showed the longest survival time, GC genotype was associated with middle-term survival, and CC genotype carriers presented the worst prognosis (Figure A, $P < 0.001$). Similarly, genotypic frequency of -1454G>A was also associated with OS of DCM patients in the dominant model (HR = 3.61, 95%CI 2.61-5.00, $P < 0.001$), the recessive model (HR = 7.58, 95%CI 3.86-14.93, $P < 0.001$), and the overdominant model (HR = 2.84, 95%CI 2.03-3.97, $P < 0.001$). Patients with GG genotype showed the longest survival time, GA genotype was associated with middle-term survival, and AA genotype carriers presented the worst prognosis (Figure B, $P < 0.001$). These associations remained significant in the multivariable survival analysis after Cox regression adjusting for potential confounding factors ($P < 0.001$).

Plasma TIM-1 levels were further determined in 30 DCM patients and 38 healthy controls. As shown in Supplemental Figure A, the plasma TIM-1 levels in DCM patients were significantly higher than that in healthy controls (259.0 pg/mL versus 149.8 pg/mL, $P = 0.035$). We further accessed the relationship between different genotypes of the two SNPs and plasma levels of TIM-1. We found that the GG genotype of -416G>C resulted in significantly lower TIM-1 production in comparison with the GC or CC genotypes ($P = 0.007$) (Supplemental Figure B). Similarly, the GG genotype of -1454G>A was also associated with significantly lower TIM-1 production than the GA or AA genotypes ($P = 0.017$) (Supplemental Fig-

Figure. Kaplan-Meier curves of DCM patients carrying different genotypes of *TIM1* SNPs. **A:** *TIM1* -416G>C SNP. **B:** *TIM1* -1454G>A SNP.

ure C). However, we detected no significant associations between BNP levels and *TIM-1* -416G>C ($P = 0.208$) or -1454G>A ($P = 0.141$) SNPs.

DISCUSSION

DCM is a complex disorder with extremely high fatality rate, despite rapid advancements in pharmacologic therapy and surgical treatment over the past 20 years, there is little improvement in its prognosis. According to several recent investigations, DCM is still one of the major causes of morbidity and mortality, especially in youngsters and those in their productive years of life.^{13,14} Thus, a better understanding of the pathogenesis of DCM is essential to further improve its survival. Human *TIM* gene family, which contains *TIM1*, *TIM3* and *TIM4*, is located on chromosome 5q23-35, a region that has been repeatedly linked with immune dysfunction, which may subsequently lead to autoimmune diseases, allergic diseases, chronic viral infections, immune rejections, and tumors.^{10,11,15}

The *TIM1* gene, also known as hepatitis A virus cellular receptor 1 (*HAVCR1*) or kidney injury molecule 1 (*KIM1*), is a vital causative agent of immunological injuries and inflammatory disorders. Previous studies have demonstrated that transient over-expression of *TIM1* or cross-linking TIM-1 with agonistic antibodies, in conjunction with T cell receptor (TCR) signal complex and CD28 stimulation, enhances T cell proliferation and cytokine production, which associates with various inflammatory disorders.¹⁶ Furthermore, Kobayashi, *et al*¹⁷ and Kim, *et al*¹⁸ reported that TIM-1 serves as a receptor for PtdSer, a signal of programmed cell death. The recognition of ptdSer by TIM-1 is essential for natural killer T (NKT) cells and macrophages to identify and respond to apoptotic cells. Although apoptosis is classically considered as a “silent death”, it has become evident recently that excessive apoptosis can be highly immunogenic, with the ability to elicit strong immunologic responses and heavy inflammatory injuries.¹⁹ Moreover, certain *TIM1* polymorphisms were demonstrated to be related to immunological disorders such as asthma,^{20,21} allergic rhinitis,²² atopic dermatitis,²³ rheumatoid arthritis,^{24,25} myasthenia gravis,²⁶ HAV infection and its associated acute liver failure,²⁷ persist hepatitis C virus (HCV) infection,²⁸ and filovirus infection²⁹ in different populations. Hence, we surmised that the *TIM1* gene may be a potential candidate marker for DCM susceptibility and prognosis.

In the present study, we investigated the associations between two SNPs located in the promoter region of the *TIM1* gene and DCM in a Han Chinese population. Although promoter region polymorphisms cannot change the coding sequence of a specific gene, they are found to affect initiation and promotion of gene transcription, and thus may be pathogenic. Previous studies showed that *TIM1* -416G>C SNP was associated with susceptibility to asthma and allergic rhinitis,^{20,22} and *TIM1* -1454G>A SNP was associated with susceptibility to allergic rhinitis and rheumatoid arthritis.^{22,24,25} In addition, luciferase reporter assay and allele-specific transcript quantification analysis demonstrated that base substitution in both sites can lead to an increased transcriptional activity of the *TIM1* gene.^{30,31} To the best of our knowledge, the current research is the first attempt to ascertain whether certain *TIM1* polymorphisms are associated with DCM susceptibility and prognosis. We found that the genotypic frequencies of the two SNPs genotyped were associated with DCM susceptibility in the codominant, dominant, and overdominant models. They were also associated with OS of DCM patients in the dominant, recessive, and overdominant models. Furthermore, the C allele of -416G>C as well as the A allele of -1454G>A were associated with an increased susceptibility to DCM, and the CC genotype of -416G>C as well as the AA genotype of -1454G>A were associated with the worst prognosis of DCM patients. Moreover, plasma levels of TIM-1 were elevated in DCM patients. Also, the GG genotype of -416G>C showed lower plasma TIM-1 levels than the GC or CC genotypes. Similarly, the GG genotype of -1454G>A also demonstrated lower plasma TIM-1 levels compared with the GA or AA genotypes. The C allele of -416 G>C and A allele of -1454G>A were associated with the highest TIM-1 production.

Overall, based on our findings, -416G>C and -1454G>A SNPs of the *TIM1* gene may predispose to the pathogenesis of DCM by up-regulating TIM-1 protein production. However, future studies are needed to better elucidate the underlying mechanisms of the associations observed between the two SNPs genotyped and DCM since the sample size of the present ELISA assessment was relatively small, which precluded us from drawing definite conclusions.

Conclusion: In conclusion, the present study suggests that *TIM1* -416G>C and -1454G>A SNPs are associated with DCM susceptibility and prognosis in this Han Chinese population. However, further studies with larger sample sizes are warranted to confirm the significance of these associations and

better clarify the mechanisms involved. Finally, given its highly polymorphic characteristic, future investigations are needed to explore the potential roles of other *TIM1* polymorphisms in the pathogenesis of DCM.

DISCLOSURE

The authors report no conflicts of interest regarding the content herein.

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SUPPLEMENTAL FILES

Supplemental Tables I, II
Supplemental Figure
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